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Using Protein Synthesis Inhibitors to Establish the Phylogenetic Relationships of the Sulfolobales Order

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Abstract. The sensitivity of the cell-free protein synthesis systems from *Acidanus brierleyi, Acidianus infernus,* and *Metallosphaera sedula,* members of the archaeal order Sulfolobales, to 40 antibiotics with different specificities has been studied. The sensitivity patterns were compared to those of *Sulfolobus solfataricus* and other archaeal, bacterial, and eukaryotic systems. The comparative analysis shows that ribosomes from the sulfolobales are the most refractory to inhibitors of protein synthesis described so far. The sensitivity results have been used to ascertain in phylogenetic relationships among the members of the order Sulfolobales. The evolutionary significance of these results are analyzed in the context of the phylogenetic position of this group of extreme thermophilic microorganisms.

Key words: Sulfolobales — Protein synthesis — Antibiotics — Ribosomal function

Introduction

According to Vazquez (1979), protein synthesis inhibitors have been classified into three groups: (1) specific inhibitors of bacterial ribosomes, (2) specific inhibitors of eukaryotic ribosomes, and (3) inhibitors which interfere with both bacterial and eukaryotic ribosomes (universal inhibitors). Since the discovery of archaeal microorganisms, studies have shown that the ribosomes from members of this domain exhibit a heterogeneous sensitivity to protein synthesis inhibitors (Hilpert et al. 1981; Böck et al. 1993; Hummel et al. 1985; Cammarano et al. 1985; Sanz et al. 1987, 1991; Altamura et al. 1988).

The phylogenetic merit of comparative ribosomal functional studies using protein synthesis inhibitors has received much study; the clusters obtained are similar to the phylogenetic trees generated with 16S rRNA sequences, validating their phylogenetic significance (Amils and Sanz 1986; Oliver et al. 1987; Amils et al. 1989, 1990).

Recent studies have led to the suggestion that hyperthermophilic microorganisms are good candidates to be considered primitive life forms. Many hyperthermophilic microorganisms are found at the base of rooted phylogenetic trees, implying that the common ancestor of extant life exhibited this characteristic (Stetter 1993; Woese and Pace 1993).

The sensitivity patterns of cell-free protein synthesis systems from three extreme thermophilic archaea belonging to the Sulfolobales order, *A. brierleyi, A. infernus,* and *M. sedula,* to 40 ribosomal inhibitors are compared to those of other archaeal, bacterial, and eukaryotic systems. The remarkable insensitivity exhibited by the hyperthermophilic archaeal ribosomes to functional inhibitors is analyzed in the context of evolutionary relationships.

Materials and Methods

Strains and Culture Conditions. Acidianus brierleyi DSM 1651 and *Acidianus infernus* DSM 3191 were grown according to Segerer et al.

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(1986). *Metallosphaera sedula* DSM 5348 was grown as described (Huber et al. 1989).

Poly(U)-Directed Polyphenylalanine Synthesis. Cell-free systems were prepared following the procedure of Cammarano et al. (1985), and polyphenylalanine synthesis directed by poly(U) was carried out according to the procedure described for *Sulfolobus solfataricus* (Cammarano et al. 1985) with the following modifications: (1) the NH_4Cl concentration for *M. sedula* was 25 mM and (2) the incubation temperature for the *A. brierleyi* and *M. sedula* systems was 65°C. Soluble factors (S-100 fraction) from *S. solfataricus* were used in all incubations due to its high polymerization efficiency and to facilitate comparative studies.

Statistical Methods. Statistical analysis was according Amils et al. (1989). Pairwise values were used as input to construct a quantitative matrix which was processed using the BMDP package (Dixon et al. 1983). The P2M subprogram (cluster analysis of cases) using euclidian distance was employed to find relationships among microorganisms. Simple-linkage and centroid-linkage cluster methods were used. The statistical package BIOSYS-1 (Swofford and Selander 1981) was used to obtain phylogenetic trees following Wagner's method (Swofford 1981), using the UPGMA clustering method and the following coefficients: modified Rogers distance, Cavalli-Sforza and Edwards distance, chord distance, arc distance, and "E" distance (Swofford and Selander 1981).

Results and Discussion

Protein synthesis cell-free systems for *A. brierleyi, A. infernus,* and *M. sedula* have been optimized in order to perform a comparative study of sensitivity to 40 elongation inhibitors with different structural, domain, and functional specificities. The results were compared with those obtained for *S. solfataricus* ribosomes, another member of the Sulfolobales order studied earlier (Cammarano et al. 1985), and other archaeal, bacterial, and eukaryotic ribosomal systems. The insensitivity of ribosomes from the Sulfolobales order to protein synthesis inhibitors is remarkable (Table 1), suggesting a strong functional correlation between the protein synthesis machinery of these extreme thermophilic archaea.

Among specific inhibitors of bacterial ribosomes (group I antibiotics) only the aminoglycoside neomycin (Fig. 1a) inhibits at low concentration. Other aminoglycosides, tobramycin and paromomycin, as well as thiostrepton and virginiamycin M, are partial inhibitors at high concentration ($IC_{50} = 5 \cdot 10^{-4}$–$10^{-3}$ M), which may be due to secondary effects.

Among specific inhibitors of eukaryotic ribosomes (group II antibiotics) only α-sarcin (Fig. 1b) and the related toxins mitogillin and restrictocin inhibit protein synthesis of the Sulfolobales ribosomes, although with lower efficiency than in the eukaryotic reference system.

Of the universal antibiotics, which interfere with both bacterial and eukaryotic ribosomes (group III inhibitors), only edeine inhibits all the Sulfolobales protein synthesis systems analyzed (Fig. 1c). The peptidyltransferase inhibitors sparsomycin (Fig. 1d), puromycin, and anthelmycin are not effective in some of the systems used (Table 1). Sensitivity of *A. brierleyi* ribosomes to sparsomycin and *A. infernus* ribosomes to puromycin, with similar sensitivities to those of the bacterial and eukaryotic reference systems (Table 1), should be underlined.

In general, ribosomes from both *Acidianus* species show similar inhibitory patterns, with *M. sedula* being the most antibiotic-insensitive ribosomal system analyzed. In comparative analyses of antibiotic sensitivities, the extreme insensitivity of Sulfolobales ribosomes is one of the most important characteristics.

The sensitivity data obtained has been used to generate phenograms and phylogenetic clusters. The results obtained are coherent regardless of the algorithm used to analyze them. The phylogenetic tree displayed in Fig. 2 shows that *S. solfataricus* and *M. sedula* form one cluster. A second cluster is formed with both *Acidianus* species, although with a low similarity coefficient. The use of sensitivity data from other archaeal, bacterial, and eukaryotic systems allowed the derivation of the correspondent phenograms. Figure 3 shows the strong correlation exhibited by the Sulfolobales ribosomes when they are compared against other ribosomes. The phenograms obtained agree with the phylogenetic clusters generated using 16S rRNA sequence analysis (Woese et al. 1990).

Although it can be argued that our conclusions are based on negative characteristics, we note that insensitivity toward ribosomal inhibitors in protein synthesis cell-free systems has strong phylogenetic value, e.g., the absolute insensitivity of all the eukaryotic ribosomes to specific bacterial inhibitors and the converse insensitivity of bacterial ribosomes to specific eukaryotic inhibitors (Table 1). Many have specific insensitivity mechanisms, such as the sensitivity of eukaryal and archaeal ribosomes to streptomycin, which has been correlated to a change of C for U in 16/18S rRNA (base 912 in the *E. coli* system) (Montandon et al. 1986); the insensitivity of bacterial and archaeal ribosomes to erythromycin, which has been correlated to an A-to-G change (*E. coli* position 2058) in the otherwise-conserved region of domain V of 23/28S rRNA associated with peptidyltransferase activity (Sor and Fukuhara 1984); and the differing sensitivity of archaeal and eukaryal vs bacterial ribosomes to α-sarcin, which correlates to an A-to-C change (*E. coli* residue 2666) in an extremely conserved region close to the 3′-end of 23/28S rRNA (Wool 1984; Amils and Sanz 1986; Casquero and Amils, unpublished).

We also note that the insensitivity of the Sulfolobales ribosomes is not related to the ecological conditions under which these hyperthermophilic archaea grow. Appropriate controls performed with thermophilic and hyperthermophilic bacteria show that their ribosomes have a sensitivity pattern similar to the correspondent mesophilic bacteria (Cammarano et al. 1985), which strongly suggests that ribosomal insensitivity of hyperthermo-

Table 1. Inhibition of protein synthesis in Sulfolobales ribosomes produced by different antibiotics[a]

Antibiotics	*A. brier.*	*A. infer.*	*M. sedula*	*S. solfa.*	*E. coli*	*B. stear.*	*S. cerev.*
Group I							
Althiomycin	−	−	−	−	++	++	−
Carbomycin A	−	−	−	−	++	++	−
Gentamicin	−	−	−	−	++	++	±
Griseoviridin	−	−	−	−	++	++	−
Kanamycin	±	±	−	−	++	++	±
Neamine	+	−	−	±	++	++	±
Neomycin	+	±	±	+	++	++	±
Paromomycin	±	±	−	±	++	++	±
Ribostamycin	−	−	−	−	++	++	−
Streptomycin	−	±	±	−	++	++	−
Thiostrepton	±	±	±	±	++	++	−
Tylosin	−	−	−	−	++	++	−
Tobramycin	±	±	±	±	++	++	±
Viomycin	±	−	−	−	++	++	−
Virginiamycin M	±	±	±	−	++	++	−
Group II							
Anisomycin	−	±	−	−	−	−	++
Bruceantin	−	−	−	−	−	−	++
Cycloheximide	−	−	−	−	−	−	++
Cryptopleurine	−	−	−	−	−	−	++
Heamanthamine	−	−	−	−	−	−	++
Harringtonine	−	−	−	−	−	−	++
Mitogillin	±	−	±	−	±	±	++
Narciclasine	−	−	−	−	−	−	++
Pretazetine	−	±	−	−	−	−	++
Restrictocine	±	−	±	−	±	±	++
α-Sarcin	±	±	±	±	±	−	++
Streptimidone	−	±	−	−	−	−	++
Streptovitacin	−	±	−	−	−	−	++
Toxin T2	−	±	−	−	−	−	++
Tubulosine	−	±	−	−	−	−	++
Tylophorine	−	±	−	−	−	−	++
Group III							
Amicetin	−	−	−	−	++	++	±
Anthelmycin	+	+	−	−	++	++	++
Blasticidin S	±	±	−	−	++	±	++
Edein	+	+	±	+	++	+	++
Fusidic acid	−	−	−	−	+	++	++
Hygromycin B	±	±	−	−	++	++	++
Puromycin	±	++	−	−	++	++	++
Sparsomycin	++	±	−	+	++	++	++
Tetracycline	±	+	−	−	++	++	++

[a] Group I: bacterial-targeted antibiotics; group II: eukaryotic-targeted antibiotics; and group III: antibiotics affecting both bacteria and eukarya. Symbols: ++: similar inhibitory profile to that of the reference system; +: one order of magnitude less active than the reference system; ±: two orders of magnitude less active than the reference system; −: no activity at the maximun concentration assayed. *A. brier.: Acidianus brierleyi; A. infer.: Acidianus infernus; M. sedula: Metallosphaera sedula; S. solfa.: Sulfolobus solfataricus; E. coli: Escherichia coli; B. stear.: Bacillus stearothermophilus; S. cerev.: Saccharomyces cerevisiae*

philic archaea is related to their phylogenetic characteristics rather than to the conditions in which the microorganisms develop.

The remarkable insensitivity of Sulfolobales ribosomes to translation inhibitors poses an interesting question regarding the evolution of ribosomal functions. The Crenarchaeota are good candidates for the most ancestral phenotype because of (1) their hyperthermophilic character (Woese and Pace 1993); (2) their relatively small genome size (Noll 1989; Yamagishi and Oshima 1990); (3) the ability of their tRNAs to replace endogenous tRNAs from protein cell-free systems from the three domains (universal adapters) (Amils et al., unpublished results); and (4) their energy-generating mechanisms (S^0-chemolithoautotrophy) (Wächterhäuser 1990; Stetter 1993). The insensitivity of their translational apparatus to protein synthesis inhibitors suggests a primitive ribosome, lacking binding sites for most known antibiotics. The binding sites would presumably have accumulated at later stages of evolution. Maximum parsimony

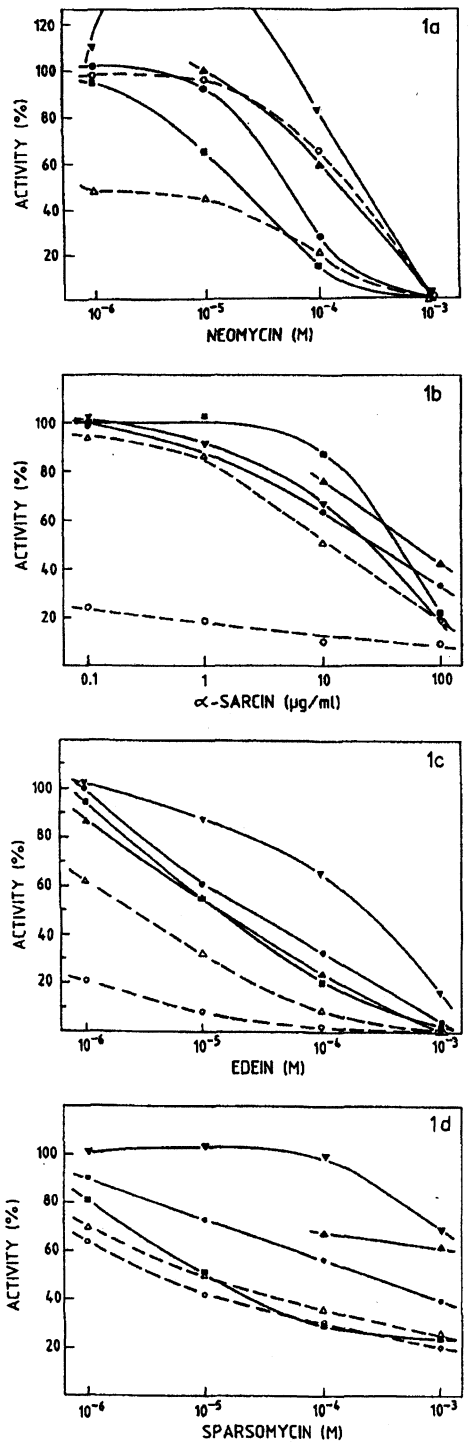

Fig. 1. Effect of selected inhibitors on phenylalanine incorporation directed by poly(U) in cell-free systems of the Sulfolobales. The protein synthesis conditions for the different systems are those described in the Materials and Methods section. *E. coli* and *S. cerevisiae* were used as reference systems. Symbols: ●, *S. solfataricus;* ■, *A. brierleyi;* ▲, *A. infernus;* ▼, *M. sedula;* △, *E. coli;* ○, *S. cerevisiae.*

analysis of the sensitivity data supports this model, assigning to the progenote only partial sensitivity to some inhibitors of the group III antibiotics (universal inhibitors). A careful analysis of the intersection of structural

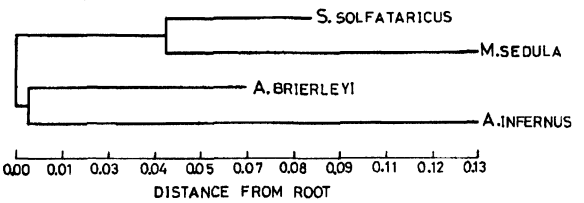

Fig. 2. Phylogenetic tree of the Sulfolobales group of archaea obtained by Wagner parsimony analysis of their ribosomal inhibition data. The functional data used are those of Table 1. The BIOSYS-1 program (UPGMA clustering method and modified Rogers distance as coefficient) was used for the statistical analysis.

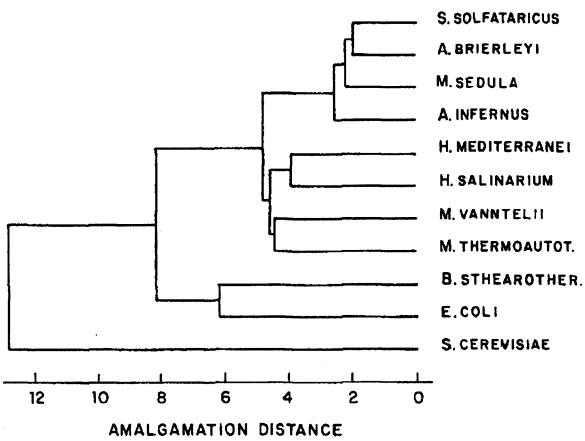

Fig. 3. Phenetic relationships among archaea, bacteria, and eukaryotes using antibiotic sensitivity data. The functional data used are those from the inhibition curves of the present work and from *H. salinarium* and *H. mediterranei* (Sanz et al. 1993); *M. thermoautotrophicum* and *M. vannielii* (Sanz, Hummel, Böck, and Amils, unpublished results); and *E. coli, B. stearothermophilis,* and *S. cerevisiae* (Cammarano et al., 1985). The inhibition curves were quantified using the algorithm described in Amils et al. (1989). The BMDP program (P2M subprogram and the single linkage clustering methods) was used for the statistical analysis.

and functional data and the use of powerful dissection techniques like footprinting and site-directed mutagenesis using different ribosomes from different phylogenetic domains should provide insight into this fundamental paradigm of evolutionary biology.

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